REMARKS

Claims 88, 90, 91, 97, 99, 105, and 106 were canceled, and new claims 107-188 added. New independent claims 107, 112, and 119 are supported by the data shown in Figs. 2A, 2B, and 3 as well as by disclosure on page 9, lines 9-30 of the specification; page 13, lines 28-32 of the specification; page 16, lines 1-31 of the specification; and page 76, line 1, to page 80, line 5, of the specification. Claims 112 and 119 are further supported by disclosure on page 70, line 16, to page 72, line 4, of the specification. Dependent claims 108-111, 113-116, and 122-125 are supported by disclosure on page 21, line 16, to page 22, line 6, of the specification. Dependent claims 117 and 118 are supported by disclosure on page 13, lines 2-7 of the specification. Dependent claims 120 and 121 are supported by disclosure on page 8, lines 9-17 of the specification and on page 21, line 16, to page 22, line 6, of the specification.

No new matter has been added by this amendment.

Double Patenting

Claims 97 and 99 were provisionally rejected for double patenting in view of claims 7 and 9 of copending application USSN 08/937,755 (the '755 application). Claims 97 and 99 have been canceled; therefore, this rejection should be withdrawn.

Claims 90, 91, 105, and 106 were provisionally rejected for double patenting in view of claims 1, 3-7 and 9 of copending application the '755 application. Claims 90, 91, 105, and 106 have been canceled; therefore, this rejection should be withdrawn.

The claims of the '755 application are drawn to methods of treating amyotrophic lateral sclerosis and spinal injury. The new claims are drawn to methods of stimulating N-CAM production and decreasing neuronal cell death using morphogens which stimulate N-CAM

production. Therefore, Applicants submit that the new claims are patentably distinct over the claims of the '755 application.

35 U.S.C. 102

Claims 90-91 and 105-106 were rejected for anticipation by Harland et al. The rejected claims have been canceled.

Harland et al. described a morphogen, dor3, which allegedly has a sequence which at least 70% homologous to the C-terminal seven cysteine domain of OP-1. The new claims specifically recite morphogens which stimulate N-CAM production. Dependent claims require that the morphogens contain specific amino acid sequences defined by SEQ ID NO: 5 or 6. Harland does not anticipate the new claims because Harland does not disclose or suggest that dor3 stimulates N-CAM production, nor is there any evidence that the sequence of dor3 is identical to amino acids 38-139 of SEQ ID NO:5 or 6, as is now required by the new claims.

35 U.S.C. 112

Claims 88, 90-91 and 105-106 were rejected for new matter/lack of written description.

As is discussed above, the rejected claims were canceled. New claims 107-125 are supported by the specification as recited above (in the Remarks section of this submission).

The claims were also rejected for overbreadth. On page 6, lines 2-7 of Paper No. 18, the Examiner states:

> The specification, while enabling for claims limited to methods of using OP-1 of claims SEQ ID NO:2 to induce N-CAM and L1 expression in NG-108 cells in vitro, does not reasonably provide enablement for "treating/preserving motor function/restoring motor function" in a mammal afflicted with ALS/spinal cord injury, or for using structurally uncharacterized morphogens or biologically functional equivalents thereof to accomplish such.

The amended claims are drawn to methods of stimulating production of N-CAM in neuronal tissue (claim 112 and those which depend from it) and methods of decreasing neuronal cell death (claims 112 and 119 and those which depend from them).

Example 6 describes N-CAM production by NG108 cells (Figs. 2A-B). Fig. 3 shows increased neuronal cell aggregation, an indication of neuronal cell viability and integrity. Applicants submit that NG108 cells are an art-recognized model for neuronal cell function. In fact, NG108 cells have been used by those skilled in the art as a model for neuronal cell function to study neurodegenerative diseases (e.g., Gangliosidosis, Alzheimer's Disease and Parkinson's Disease) as well as to study the effect of chemical and physical injury or trauma. For example, as far back as 1987 in a paper entitled "Inactivation of GM1-gangliosie beta galactosidase by a specific inhibitor: a model for ganglioside storage disease", NG108 cells were used to establish an *in vitro* model for human ganglioside storage disease (Singer et al., 1987, Ann. Neurol. 21:497-503 Appendix A), and in 1992 in a paper entitled "Ethanol-responsive gene expression in neural cell cultures", NG108 cells were used as a model for ethanol-induced injury and changes in gene expression (Miles et al., 1992, Biochim. Biophys. Acta 1138:268-274 Appendix B). In 1992 in a paper entitled "Alzheimer disease brain extract stimulates branching of laminin-mediated neuronal processes", the cells were used to study neurotrophic effects of Alzheimer's Disease brain extracts on neuronal cells (Kittur et al., Alzheimer Dis. Assoc. Disord. 6:103-110 Appendix C), and in 1993 in a paper entitled "Dopamine transporter expression confers cytotoxicity to low doses of the parkinsonianism-inducing neurotoxin 1-methyl-4 phenylpyridinium", the cells were used to establish a model for Parkinson's Disease. (Pifl et al., J. Neurosci. 13:4246-4253 Appendix D). In 1996 in a paper entitled "Acute Alterations in $[Ca^{2+}]i$ in NG108 cells subjected to high strain rate deformation and chemical hypoxia: an *in*

vitro model for neural trauma", NG108 cells were used in an *in vitro* model for neuronal injury (Cargill et al., 1996, J. Neurotrauma 13:395-407 Appendix E). Since the filing of the application, hundred of research papers have been published using NG108 cells as a model for neuronal function and to study various neurological disease states.

Since the priority date of the present application, induction of N-CAM production by morphogens, e.g., BMP2, BMP4 (a.k.a., BMP2B; see page 23, line 15, of the specification), BMP-5, BMP-6, and OP-1 (a.k.a. BMP-7), has been confirmed (see, e.g., Perides et al., 1994, J. Biol. Chem. 269:765-770; copy provided in Appendix F). Therefore, one skilled in the art would readily acknowledge that data obtained *in vitro* using NG108 cells are predictive of the behavior of neuronal cells *in vivo*

Moreover, morphogen enhancement of neuronal cell survival was evaluated using primary neuronal cells, e.g., primary cultures of striatal basal ganglia isolated from the substantia nigra of adult rat brains. These data, which are described in Example 3 (page 70, line16, to page 72, line 4, of the specification) indicate that cell death decreased significantly in a dose-dependent manner in the presence of a morphogen. In contrast, in the absence of the morphogen, the cultured primary cells dissociated and underwent cell necrosis.

In view of the description of the methods provided in the specification and the collective data regarding N-CAM production and the enhancement of cell survival *in vitro*, Applicants submit that the methods, as now claimed, comply with the requirements for enablement required by the statute.

CONCLUSION

Applicants file concurrently herewith a petition for revival, together with a check for $620.00 to cover the fee pursuant to 37 C.F.R. § 1.37(b). The Commissioner is hereby authorized to charge any deficiency of same, or credit any overpayment, to Deposit Account No. 50-0311 (Reference No. 00960-504 FWCCN2).

On the basis of the foregoing amendments and remarks, Applicants respectfully submit that the pending claims are in condition for allowance. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,

Ivor R. Elrifi, Reg. No. 39,529
Ingrid A. Beattie, Reg. No. 42,306
Attorneys for Applicants
c/o MINTZ, LEVIN
One Financial Center
Boston, Massachusetts 02111
Tel: (617) 542-6000
Fax: (617) 542-2241

Date: June 29, 2001

APPENDIX

107. A method for stimulating a Neural Cell Adhesion Molecule (N-CAM) production in a neuronal cell, comprising contacting said neuronal cell with a morphogen selected from the group consisting of an OP-1 polypeptide, a CBMP2A polypeptide, a CBMP2B polypeptide, a BMP-5 polypeptide, and a BMP-6 polypeptide.

108. The method of claim 107, wherein said OP-1 polypeptide comprises the amino acid sequence of residues 38-139 of SEQ ID NO:5.

109. The method of claim 107, wherein said OP-1 polypeptide comprises the amino acid sequence of residues 38-139 of SEQ ID NO:6.

110. The method of claim 107, wherein said OP-1 polypeptide comprises the amino acid sequence of SEQ ID NO:5.

111. The method of claim 107, wherein said OP-1 polypeptide comprises the amino acid sequence of SEQ ID NO:6.

112. A method for decreasing neuronal cell death associated with a neuropathy, comprising contacting said neuronal cell with a morphogen which stimulates N-CAM production, said morphogen being selected from the group consisting of OP-1 polypeptide, a CBMP2A polypeptide, a CBMP2B polypeptide, a BMP-5 polypeptide, and a BMP-6 polypeptide.

113. The method of claim 112, wherein said OP-1 polypeptide comprises the amino acid sequence of residues 38-139 of SEQ ID NO:5.

114. The method of claim 112, wherein said OP-1 polypeptide comprises the amino acid sequence of residues 38-139 of SEQ ID NO:6.

115. The method of claim 112, wherein said OP-1 polypeptide comprises the amino acid sequence of SEQ ID NO:5.

116. The method of claim 112, wherein said OP-1 polypeptide comprises the amino acid sequence of SEQ ID NO:6.

117. The method of claim 112, wherein said neuropathy is amyotrophic lateral sclerosis.

118. The method of claim 112, wherein said neuropathy is selected from the group consisting of Alzheimer's Disease, Huntington's chorea, and multiple sclerosis.

119. A method for decreasing neuronal cell death associated with a chemical or physical injury, comprising contacting said neuronal cell with a morphogen which stimulates N-CAM production, said morphogen being selected from the group consisting of OP-1 polypeptide, a CBMP2A polypeptide, a CBMP2B polypeptide, a BMP-5 polypeptide, and a BMP-6 polypeptide.

120. The method of claim 119, wherein said neuronal cell is contacted with said morphogen prior to said injury.

121. The method of claim 119, wherein said neuronal cell is contacted with said morphogen after said injury.

122. The method of claim 119, wherein said OP-1 polypeptide comprises the amino acid sequence of residues 38-139 of SEQ ID NO:5.

123. The method of claim 119, wherein said OP-1 polypeptide comprises the amino acid sequence of residues 38-139 of SEQ ID NO:6.

124. The method of claim 119, wherein said OP-1 polypeptide comprises the amino acid sequence of SEQ ID NO:5.

125. The method of claim 119, wherein said OP-1 polypeptide comprises the amino acid sequence of SEQ ID NO:6.

1: *Ann Neurol* 1987 May;21(5):497-503 Related Articles, Books, LinkOut

Inactivation of GM1-ganglioside beta-galactosidase by a specific inhibitor: a model for ganglioside storage disease.

Singer HS, Tiemeyer M, Slesinger PA, Sinnott ML

This study was designed to establish an in vitro model with biochemical and morphological similarities to the human neurodegenerative disease GM1 gangliosidosis. Utilizing a specific inactivator of the lysosomal enzyme GM1-ganglioside beta-galactosidase (beta-D-galactopyranosylmethyl-p-nitrophenyltriazene [beta-GalMNT]) and neuroblastoma X glioma hybrid cells (NG108-15), we suppressed beta-galactosidase activity for up to 72 hours. Coincidental with suppression of this enzyme to levels less than 1% of control, we found up to a nine-fold accumulation of its substrate, the GM1-ganglioside, and the ultrastructural appearance of membranous cytoplasmic bodies. beta-GalMNT treatment suppressed growth but had little effect on the specific activity of choline acetyltransferase, lactate dehydrogenase, or other lysosomal enzymes including galactosylceramidase. This model should permit studies of the neurophysiological effects of increased ganglioside accumulation and their reversibility.

PMID: 3035998

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Appendix B

1: *Biochim Biophys Acta* 1992 Apr 14;1138(4):268-74 Related Articles, Books

Ethanol-responsive gene expression in neural cell cultures.

Miles MF, Diaz JE, DeGuzman V

Department of Neurology, University of California School of Medicine, San Francisco.

We have studied the molecular mechanisms underlying neuronal adaptation to chronic ethanol exposure. NG108-15 neuroblastoma cells were used to perform a detailed analysis of ethanol-induced changes in neuronal gene expression. High resolution, quantitative two-dimensional (2-D) gel electrophoresis of in vitro translation products showed both dose-dependent increases and decreases in specific mRNA abundance following treatment with ethanol at concentrations seen in actively drinking alcoholics (50-200 mM). Dose response curves for representative members of the increasing or decreasing response groups had very similar profiles, suggesting that similar mechanisms may regulate members of a response group. Some mRNAs that increased with ethanol treatment appeared identical to species induced by heat shock while other mRNAs were only induced by ethanol. We conclude that chronic ethanol exposure can produce specific coordinate changes in expression of neuronal mRNAs, including some members of the stress protein response. However, the overall pattern of ethanol-responsive gene expression is distinct from the classical heat shock subgroup of stress proteins response. Changes in gene expression and specifically, mechanisms regulating a subset of stress protein expression, could be an important aspect of neuronal adaptation to chronic ethanol seen in alcoholics.

PMID: 1562614

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1: *Alzheimer Dis Assoc Disord* 1992 Summer;6 (2):103-10

Alzheimer disease brain extract stimulates branching of laminin-mediated neuronal processes.

Kittur SD, Endo H, Adler WH, Martin GR, Markesbery WR, Kleinman HK, Weeks BS

Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, Maryland.

Patients with Alzheimer disease (AD) suffer mental deterioration associated with neurofibrillary tangle and senile plaque formation in the brain. Here we have determined the effects of brain extracts from normal and from AD patients on neuronal process formation by a pheochromocytoma (PC-12) and a neuroblastoma x glioma hybrid cell line (NG108-15). PC12 cells show a dose-related stimulation of branching of neuronal processes by AD brain extracts with cells cultured on a laminin substrate. The neurotrophic effects of extracts of AD brains may be related to the abnormal sprouting and neurofibrillary tangle formation observed in the brain in this disorder.

PMID: 1389079

Appendix D

1: *J Neurosci* 1993 Oct;13(10):4246-53

Dopamine transporter expression confers cytotoxicity to low doses of the parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium.

Pifl C, Giros B, Caron MG

Howard Hughes Medical Institute Laboratories, Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710.

The uptake of 1-methyl-4-phenylpyridinium (MPP+), the active metabolite of the parkinsonism-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was studied in various mammalian cell lines transfected, respectively, with the cloned human and rat dopamine transporters, and compared with rat striatal synaptosome preparations. Only in neuronally derived cell lines such as NG108-15, NS20Y, and SK-N-MC cells did MPP+ have a KM for the cloned transporters comparable to that of dopamine as seen in rat striatal synaptosomes. In non-neuronally derived cells such as COS-7, CHO, and Ltk- cells transiently or permanently expressing the transporters, the KM of MPP+ was at least 10-fold higher. The permanent expression of either the cloned human or rat dopamine transporters conferred to SK-N-MC cells susceptibility to the cytotoxic effects of low concentrations of MPP+. The extent of this effect was dependent on the expression level of the dopamine transporters and could be specifically antagonized by the catecholamine uptake inhibitor mazindol. There were no significant differences in the susceptibility to MPP+ of cells expressing similar levels of either the human or rat dopamine transporter. The demonstration for the first time of a quantitative relationship between the cellular expression of the plasma membrane transporter and the extent of the cytotoxic effects of MPP+ suggests that known differences in vulnerability of various brain regions to MPP+ cytotoxicity might be related to their actual content of dopamine uptake sites.(ABSTRACT TRUNCATED AT 250 WORDS)

PMID: 8410185

Appendix E

1: *J Neurotrauma* 1996 Jul;13(7):395-407

Acute alterations in [Ca2+]i in NG108-15 cells subjected to high strain rate deformation and chemical hypoxia: an in vitro model for neural trauma.

Cargill RS, Thibault LE

Department of Bioengineering, University of Pennsylvania, Philadelphia 19104, USA.

The short-term (less than 2 min) alterations in the intracellular free calcium concentration in differentiated NG108-15 (neuroblastoma cross glioma) cells exposed to dynamic mechanical deformation with and without superimposed chemical hypoxia were determined. A previously developed device, modified for these studies, was used to apply deformations at a magnitude and rate representative of those experienced by neural tissue in Traumatic Brain Injury. Chemical hypoxia was imposed using a combination of 2-deoxy-D-glucose and salicylate, anaerobic and aerobic metabolic blockers, respectively. Real time measurement of intracellular free calcium concentration using Fura-2 and a custom epifluorescence microscopy system provided a quantitative index of cell response. At high rates of deformation (approximately 10 sec-1), increases in intracellular free calcium concentration were exponentially related to the magnitude of the applied deformation. Chemical hypoxia had no effect on this acute response. At low rates of deformation, small increases in intracellular free calcium concentration were independent of the magnitude of the deformation. These findings indicate that strategies for reducing severity of TBI should focus on minimizing the rate of deformation of neural cells. Together with data from animal, physical, and finite element models, these data can be employed in the development of physiologic injury tolerance criteria for the whole head.

PMID: 8863195

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Appendix F

Regulation of Neural Cell Adhesion Molecule and L1 by the Transforming Growth Factor-β Superfamily

SELECTIVE EFFECTS OF THE BONE MORPHOGENETIC PROTEINS*

(Received for publication, August 2, 1993, and in revised form, September 3, 1993)

George Perides‡§, Rebecca M. Safran¶‖, Lou Ann Downing¶‖, and Michael E. Charness¶‖ **

From the ‡Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, the §Spinal Cord Injury Laboratory, Department of Veterans Affairs Medical Center, West Roxbury, Massachusetts 02132, the ¶Department of Neurology and Program in Neuroscience, Harvard Medical School, Division of Neurology, Brigham and Women's Hospital, Boston, Massachusetts 02115, and the ‖Section of Neurology, Department of Veterans Affairs Medical Center, West Roxbury, Massachusetts 02132

The transforming growth factor-β (TGF-β) superfamily plays a role in embryogenesis and regeneration. We have reported that osteogenic protein-1 (OP-1) promotes cell aggregation and induces the expression of the neural cell adhesion molecules N-CAM and L1 in proliferating neuroblastoma × glioma hybrid NG108-15 cells (Perides, G., Safran, R. M., Rueger, D. C., and Charness, M. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 10326-10330; Perides, G., Hu, G., Rueger, D. C., and Charness, M. E. (1993) *J. Biol. Chem.* 268, 25197-25205). Here we show that the structurally homologous bone morphogenetic proteins (BMP) BMP-2 and BMP-4 are 10-50-fold more potent in these actions than the subfamily comprising BMP-5, BMP-6, and OP-1 (BMP-7). In contrast, members of the TGF-β subfamily, activin-A, inhibin-A, and 29 additional growth factors and cytokines did not induce N-CAM. The addition of serum to cells growing in serum-free medium caused a concentration-dependent increase in N-CAM and L1 expression; however, serum did not potentiate the induction of N-CAM and L1 by 40 ng/ml OP-1. These findings suggest the presence in NG108-15 cells of a BMP-2/BMP-4 receptor that discriminates subtle differences in structure among homologous members of the TGF-β superfamily. An endogenous ligand for this receptor may be present in serum.

The TGF-β[1] superfamily plays an important role in the development, differentiation, and repair of diverse tissues (1). This superfamily is defined by a common structural element, a 7-9 cysteine domain in the C-terminal region of the mature protein (1). Based on sequence homology in this domain, the TGF-β superfamily has been subdivided into several subfamilies (1-6): the TGF-β subfamily (TGF-β1, -2, -3, -4, -5); the inhibin/activin subfamily (inhibin-A, inhibin-B, activin-A, and activin-B); Müllerian inhibiting substance; and the *dpp*/Vg-related subfamily (decapentaplegic complex (*dpp*) and Vgr/60A from *Drosophila melanogaster*, Vg-1 from *Xenopus laevis*, Vgr-1 and GDF-1 from mouse, osteogenic proteins-1 and 2, the bone morphogenetic proteins (BMPs) 1 through 7, and dorsalin-1). The BMP subfamily can be further subdivided into two subgroups: BMP-2/BMP-4 and BMP-5/BMP-6/BMP-7 (OP-1) (7). Various members of the *dpp*/Vg-1 subfamily are morphogenetically active in the developing nervous system and in bone. For example, the BMPs stimulate endochondral bone formation (8), but have also been identified as morphogens and differentiation factors in neural cells (3, 4, 9, 10).

Cell adhesion molecules are important mediators of cell-cell interactions during embryogenesis and tissue repair (11). Indeed, the neural cell adhesion molecule N-CAM is induced by TGF-β in 3T3 fibroblasts(12), by activin in developing limb buds (13) and by OP-1 (BMP-7) in neuroblastoma × glioma NG108-15 hybrid cells (10). These observations imply that a distinct signaling pathway may couple the TGF-β superfamily to the induction of cell adhesion molecules in mesenchymal and neural tissue. The ligand specificity of the receptor or receptors mediating this response is unknown. Here we demonstrate that the BMPs differentially induce morphological changes and increase the expression of N-CAM and L1 in NG108-15 cells, whereas the TGF-βs, activin-A, inhibin-A, and a large number of other growth factors and cytokines are inactive.

EXPERIMENTAL PROCEDURES

Materials—Rat monoclonal antibody (mAb) H28.123 against N-CAM (14) was purchased from AMAC Inc. (Westbrook, ME) and mAb 5B8 from the Developmental Studies Hybridoma Bank (Iowa City, IA). mAb 74-5H7 against L1 (15) was a generous gift from Dr. V. Lemmon, Case Western Reserve, Cleveland, OH. ^{125}I-Goat anti-rat antibody was obtained from ICN Biomedicals (Irvine, CA), and horseradish peroxidase-conjugated goat anti-mouse was from TAGO (Burlingame, CA). Fetal bovine, newborn calf, and adult bovine sera were purchased from Intergen (Purchase, NY). Epidermal growth factor (EGF) and 2.5 S nerve growth factor (NGF) were purchased from Life Technologies, Inc. Recombinant human OP-1 (hOP-1) was kindly provided by Dr. David C. Rueger, Creative BioMolecules (Hopkinton, MA) and was isolated as described (16). Generous gifts of additional recombinant growth factors were provided as follows: recombinant human BMP-2, BMP-4, BMP-5, and BMP-6 were from Dr. John Wozney, Genetics Institute (Cambridge, MA); recombinant human activin-A and inhibin-A were from Dr. Ralph Schwall, Genentech (South San Francisco, CA); *dpp* and 60A were from Dr. Michael Hoffman, University of Wisconsin (Madison, WI); and the remaining human recombinant growth factors and cytokines were from Dr. Monica Tsang, R & D Systems (Minneapolis, MN). All other chemicals were purchased from Sigma and were of reagent grade.

Cell Culture—NG108-15 cells of passages 21-30 were cultured in serum-free medium as described (10). Prior to subculture, the cells were mechanically dispersed until single. Cells were photographed at a mag-

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** To whom correspondence should be addressed: Dept. of Neurology (127), Harvard Medical School, 1400 VFW Parkway, Boston, MA 02132. Tel.: 617-325-2815; Fax: 617-323-7700 (ext. 5033).

[1] The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic proteins; *dpp*, decapentaplegic complex; EGF, epidermal growth factor; FGF, fibroblast growth factor; hOP-1, recombinant human osteogenic protein-1; IgCAM, immunoglobulin superfamily cell adhesion molecule; mAb, monoclonal antibody; N-CAM, neural cell adhesion molecule; NGF, nerve growth factor; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

nification of × 200 under phase contrast microscopy. Because BMP-treated cells grew in clusters, some × 200 fields contained clusters of large numbers of cells while others showed few; therefore, the photographs do not accurately reflect cell number, which was not influenced by BMP treatment.

Morphogenetic Actions of hOP-1—Two days after the addition of recombinant growth factors, subconfluent (<50%) cells viewed at × 100 magnification were scored for the presence of cell clusters. A cell cluster was defined as a group of three or more cells that adhered to each other along at least one-quarter of the cell diameter. Adherent pairs of cells were excluded from this definition because many consisted of post-mitotic pairs which, in the absence of growth factors, eventually separated. The percentage of cells in clusters was calculated by dividing the number of cells present in clusters by the total number of cells (150–200) in each of two randomly selected fields. Values obtained for the two fields were averaged and differed by up to 15%.

ELISA for N-CAM—Levels of N-CAM were quantitated using an ELISA as described (17, 18), with the following modifications. To determine the linearity of the assay with respect to cell number, NG108–15 cells were cultured for 3 days in 75-cm² flasks in the absence and presence of 40 ng/ml hOP-1. 18 h before assay, duplicate samples of 6,000–90,000 cells/well were plated in poly-D-lysine-coated 96-well trays in the continued presence of hOP-1. To determine the effects of growth factors on N-CAM expression, duplicate samples of cells were plated on 96-well trays at a density of 4,500 cells/well and incubated for 24–48 h in serum-free medium. Following the addition of growth factors, the medium was replaced daily. N-CAM was assayed after 3 days of treatment with growth factors. Cells were fixed by incubating twice for 30 min at 4 °C in 100 µl of ice-cold methanol, washed three times with PBS, blocked with 10% non-fat dry milk in PBS for 1 h, and washed three additional times with PBS. Cells were then incubated for 1 h at room temperature in 100 µl of PBS containing 5% fetal bovine serum supplemented with the primary antibody (1:2,000 dilution of anti-N-CAM mAb 5B8), washed three times in PBS, and incubated an additional 1 h at room temperature in 100 µl of PBS containing 5% fetal bovine serum supplemented with the secondary antibody (1:1,000 dilution of goat anti-mouse IgG and IgM conjugated to horseradish peroxidase). The cells were washed an additional three times with PBS. Color was developed by adding 100 µl of 80 mg/dl of 5-aminosalicylic acid in 0.02 M monobasic sodium phosphate, pH 6.0, containing 0.02% H_2O_2, and the optical density was measured at 490 nm. The reaction was linear with respect to cell number between 6,000 and 50,000 cells/well. Nonspecific color reaction, determined by omitting the primary antibody in duplicate wells, was subtracted from the total optical density for each sample. All values are expressed relative to control values measured on the same tray.

Western Blot Analysis—Western blots of total cell protein were performed using mAb H28.123 for N-CAM and mAb 74–5H7 for L1, as described (10, 19).

RESULTS AND DISCUSSION

Morphogenetic Activity of the BMPs in NG108–15 Cells—We showed previously that hOP-1 induces the expression of the immunoglobulin superfamily cell adhesion molecules (IgCAM) N-CAM and L1 in dividing NG108–15 cells, leading to a concentration-dependent increase in the percentage of cells present in adherent clusters and epithelioid sheets (10, 19). To determine the morphogenetic activity of other members of the TGF-β superfamily, NG108–15 cells were incubated for 2 days in serum-free medium supplemented with 1 or 10 ng/ml of various BMPs or TGF-β1 and scored for the presence of adherent clusters of cells. At 10 ng/ml, all of the BMPs induced cluster formation (Fig. 1). However, at 1 ng/ml, BMP-2 and BMP-4 were more effective in promoting cell clustering than BMP-5, BMP-6, and OP-1. In contrast, TGF-β1 was morphogenetically inactive at concentrations ranging from 0.01 to 40 ng/ml. OP-1 did not change the rate of NG108–15 cell division (19) and treatment with the BMPs did not significantly affect the protein content per well (BMP-2, 104 ± 13% control; BMP-4, 94 ± 13%; BMP-5, 91 ± 11%; BMP-6, 126 ± 14%; OP-1 (BMP-7) 96 ± 2%; n = 3–5). These data suggest that in NG108–15 cells, the morphogenetic activity of the TGF-β superfamily increases as a function of structural homology with the BMP-2/BMP-4 subfamily and is not related to effects on cell growth. These

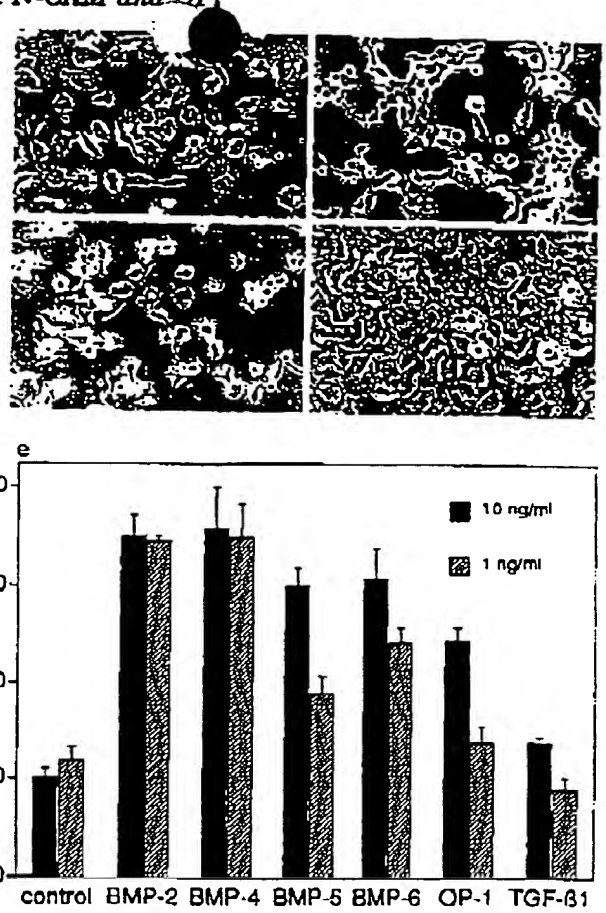


FIG. 1. **Morphological effects of the BMPs in NG108–15 cells.** Photomicrographs of NG108–15 cells cultured for 2 days in serum-free medium in the absence (*a*) and presence of 1 ng/ml of BMP-4 (*b*), 1 ng/ml BMP-6 (*c*), and 40 ng/ml TGF-β1 (*d*). Lower concentrations of TGF-β1 were also morphogenetically inactive. Note that the TGF-β-treated cells, while present at a greater density in the field shown, do not form more clusters than the control cells. *e*, NG108–15 cells were cultured in serum-free medium in the absence and presence of 1 or 10 ng/ml of the indicated recombinant human growth factors. The percentage of cells present in adherent groups of three or more cells (clusters) was determined from two subconfluent fields of at least 150 cells (see "Experimental Procedures"). The total number of cells in the two fields did not differ among control and BMP-treated cells. Shown are the mean ± S.E. percentage of cells present in clusters from three to four independent experiments. The two control values are from duplicate sets of experiments.

experiments also suggest that the morphoregulatory activity of the BMPs is mediated by a distinct receptor(s) from that which recognizes the TGF-β subfamily. Additional evidence for the existence of distinct OP-1 and TGF-β receptors derives from studies in cultured osteoblasts. Whereas both hOP-1 and TGF-β1 promote the proliferation of osteoblasts in culture, only hOP-1 stimulates markers of the osteoblast phenotype (16).

Effects of the TGF-β Superfamily on N-CAM and L1 Expression—The morphoregulatory actions of hOP-1 are mediated in part by the induction of N-CAM and L1 (10, 19). To determine whether the variable morphogenetic activity of the TGF-β superfamily is due to differential induction of IgCAMs, we cultured NG108–15 cells for 3 days in the absence and presence of members of the TGF-β superfamily and measured levels of N-CAM and L1 by ELISA and Western blot analysis. Of several antibodies tested, the best results for the ELISA detection of N-CAM were obtained using mAb 5B8, which recognizes the cytoplasmic domain present in N-CAM-140 and N-CAM-180, but not N-CAM-120. To confirm that the ELISA was linear with

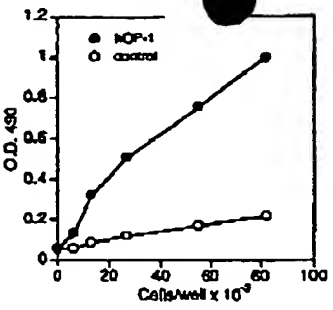


FIG. 2. **ELISA determination of N-CAM levels in control and hOP-1-treated cells.** NG108–15 cells were cultured for 3 days in T75 flasks in the absence and presence of 40 ng/ml hOP-1. 18 h before assay, cells were subcultured at the indicated densities in poly-D-lysine-coated 96-well plastic trays. Cells were fixed and N-CAM levels were determined by ELISA using mAb 5B8. Shown are the mean optical densities from duplicate samples of cells from a representative experiment that was repeated five times with similar results.

respect to cell number, we cultured NG108–15 cells at a density of 6,000–90,000 cells/well in 96-well microtiter plates and measured levels of N-CAM. Fig. 2 indicates that the ELISA was linear within a 15-fold range of cell concentrations. This finding is of interest, because N-CAM levels increase when N2A neuroblastoma cells are plated at a high density, and conditioned medium from N2A neuroblastoma cells increases N-CAM expression in N2A neuroblastoma and 3T3 fibroblast cells (12). The observation that N-CAM levels are linear with respect to NG108–15 cell numbers suggests that in contrast to N2A neuroblastoma cells, NG108–15 cells do not produce an autocrine regulator of N-CAM expression.

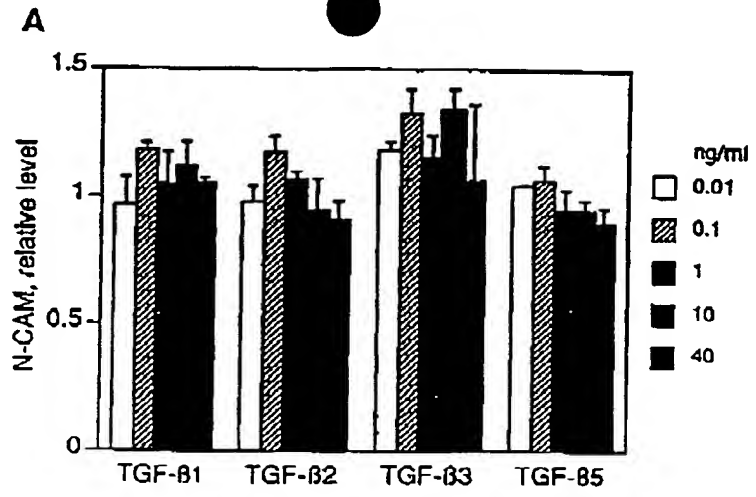
The ELISA was also effective in demonstrating a large induction of N-CAM by hOP-1 (Fig. 2), allowing us to use this rapid technique for quantitatively screening the effects of the TGF-β superfamily and other growth factors on the levels of N-CAM. None of the TGF-βs showed significant N-CAM-inducing activity (Fig. 3*a*); in contrast, the BMPs exhibited striking and differential concentration-dependent induction of N-CAM (Fig. 3*b*). Because mAb 5B8 detects only two of the three major N-CAM isoforms induced by hOP-1, the ELISA tended to underestimate the magnitude of N-CAM induction demonstrated by Western blot analysis (10). The potencies of the BMPs in inducing N-CAM correlated with their structural homology. BMP-4 showed half-maximal effectiveness at a concentration of approximately 0.2 ng/ml. The other member of this subfamily, BMP-2, was the next most potent BMP (EC_{50} ~1 ng/ml). In contrast, BMP-5, BMP-6, and OP-1 did not significantly induce N-CAM at concentrations lower than 1 ng/ml and exhibited equivalent effects at higher concentrations (EC_{50} ~10 ng/ml). Western blot analysis confirmed these results and demonstrated a comparable ordering of the BMPs in the induction of L1 (Fig. 3*c*). The fact that the potency of each BMP in inducing IgCAMs was similar to that for promoting cell clustering provides additional evidence that the morphoregulatory actions of the BMPs in NG108–15 cells are mediated by the induction of N-CAM and L1. Activin-A, inhibin-A, NGF, and EGF did not induce any of the major N-CAM isoforms or L1 (Fig. 3*c*).

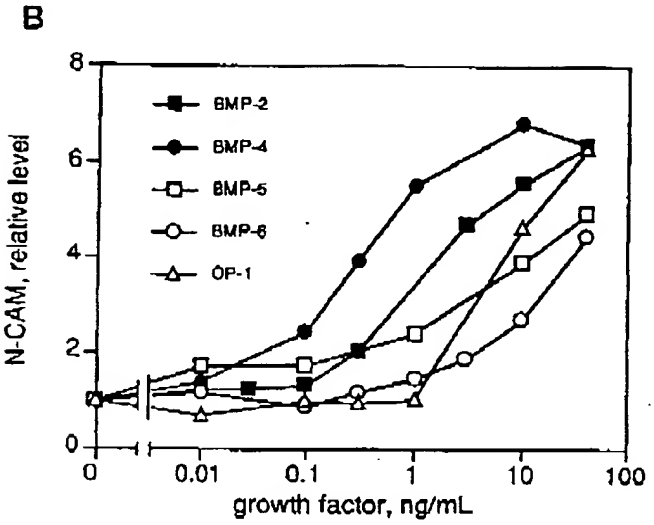
BMP-2 and BMP-4 are 92% identical in the TGF-β domain; similarly, BMP-5, BMP-6, and OP-1 (BMP-7) show 87–88% amino acid sequence identity (7). These two BMP subgroups differ only slightly from each other (57–61% identity), yet show a 10–50-fold difference in their potency for inducing N-CAM. Our results indicate that a receptor expressed in NG108–15 cells can discriminate a small difference in structure between the two BMP subgroups and fails to recognize activin-A (41% sequence identity with BMP-4), the TGF-βs (32–35% identity BMP-4), and inh [redacted] (22% identity BMP-4). Moreover, the same receptor appears to be responsible for the induction of both N-CAM and L1. A careful analysis of IgCAM-inducing activity and sequence homology among additional members of the TGF-β superfamily may allow a precise identification of the molecular domains responsible for the activation of this receptor. Interestingly, *dpp* and 60A, the *Drosophila* homologs of the BMP-2/BMP-4 (75% identity) and BMP-5/BMP-6/OP-1 (69–74% identity) subfamilies, respectively (7, 20), did not induce N-CAM expression in NG108-15 cells (not shown). This implies that the region of the protein that differs only slightly between BMP-4 and *dpp* is particularly important for activating signaling along this pathway.

Effect of Serum and Diverse Growth Factors on Cell Morphology and N-CAM Expression—Serum may contain factors that regulate IgCAM expression: growth in serum increases the expression of N-CAM and decreases the expression of L1 in N2A neuroblastoma cells (12). Small increases in N-CAM expression are also observed when serum-free myotube cultures are incubated with serum or a variety of growth factors (21). To evaluate whether serum regulates the expression of N-CAM in NG108-15 cells, we cultured cells for 3 days in 96-well trays containing serum-free medium supplemented with 0.1–10% fetal bovine, newborn calf, or adult bovine sera. Fig. 4 demonstrates that these sera caused a concentration-dependent increase in the expression of N-CAM that was inversely proportional to the age of the donor. This developmental loss of N-CAM-inducing activity in serum is consistent with previous observations that OP-1 and BMP gene expression declines during development (22, 23). The induction of N-CAM and L1 by serum led to the formation of cell clusters and multilayered cell aggregates; however, in contrast to hOP-1 (19), serum did not cause a small percentage of cells to extend neurites (data not shown). The presence in serum of thrombin and related substances that inhibit neuritogenesis (24) may have opposed the neurite promoting actions of N-CAM.

The strong IgCAM-inducing activity of the BMPs raises the question as to whether these proteins account for the IgCAM-inducing activity of serum. We addressed this question in two ways. First, using the ELISA, we screened a large and diverse number of recombinant morphogens and cytokines for N-CAM inducing activity. Table I shows that among 40 growth factors, only the BMPs exhibited significant N-CAM-inducing activity. The list of inactive factors is significant for the presence of several peptides that promote the survival, differentiation, and morphogenesis of the brain and skeleton, including NGF, EGF, platelet-derived growth factor, fibroblast growth factor-4, interleukin-6, and leukemia inhibitory factor (25, 26). Second, we asked whether the actions of serum and the BMPs are synergistic or additive. NG108–15 cells were cultured for 3 days in serum-free medium supplemented with 0.1–10% fetal bovine serum in the absence and presence of 40 ng/ml hOP-1. Fetal bovine serum produced a concentration-dependent increase in the levels of all three major isoforms of N-CAM and L1 (Fig. 4*b*); however, the induction of IgCAMs by hOP-1 was not potentiated by serum. In contrast, the N-CAM-inducing effects of serum and TGF-β are additive in NIH 3T3 cells (12). Although these experiments do not prove that BMPs are present in serum, they suggest that BMPs or factors that act similarly account for part of the IgCAM-inducing activity of serum.

Morphoregulatory and IgCAM-inducing Activity of the TGF-β Superfamily in Mesenchymal and Neural Tissue—The morphoregulatory actions of the BMPs have been well characterized in bone (8); however, an increasing body of data suggests that these proteins play an important role in the development of the nervous system. Several of the BMPs exhibit unique spatial and temporal patterns of expression in the de-





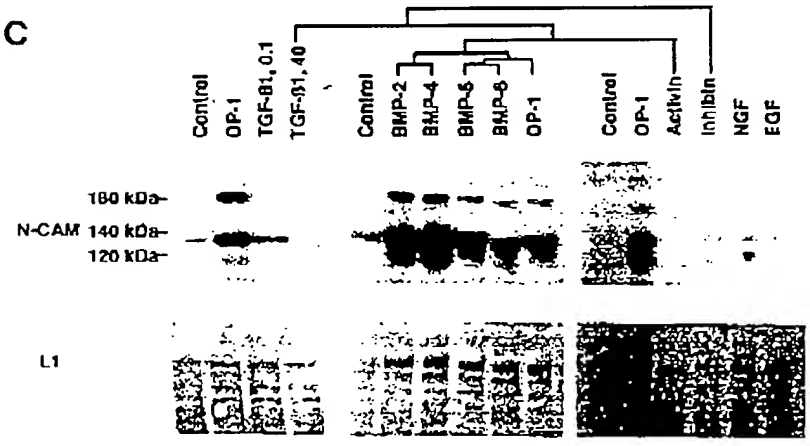


FIG. 3. Effect of the TGF-β superfamily on N-CAM and L1 expression. For ELISA determination of N-CAM, NG108–15 cells were plated at 4,500 cells/well in 96-well poly-D-lysine-coated plastic trays and incubated for 3 days in the absence and presence of the indicated concentrations of TGFβs (*A*) and BMPs (*B*). Optical densities for control values determined on the same tray were normalized to 1 and values for the morphogen-treated cells were expressed relative to control values (relative change). Shown are the mean ± S.E. (*A*) or the mean (*B*) values from three to four independent experiments. *C*, NG108-15 cells were cultured in six-well trays for 3 days in the absence (control) and presence of 10 ng/ml of BMP-2, BMP-4, BMP-5, BMP-6, and hOP-1 and 40 ng/ml of activin-A, TGF-β1, inhibin-A, nerve growth factor (NGF), and epidermal growth factor (EGF). Cells were harvested, solubilized in SDS sample buffer, and the proteins were subjected to immunoblot analysis for N-CAM (H28.123 mAb) and L1 (74-6H7 mAb). Shown are representative immunoblots from an experiment that was repeated three times with similar results. The structural homology of the TGF-β superfamily is indicated by a map of amino acid sequence identity in the C-terminal TGF-β domain, as reported by others (7, 20).

veloping nervous system, and some are known to be species homologues of developmental genes in *Drosophila* and *Xenopus*. (27, 28). BMP-4 mRNA is expressed transiently in the floorplate of the diencephalon adjacent to Rathke's pouch, where it is speculated to play an inductive role during pituitary development (29). BMP-4 also stimulates differentiation in PC12 cells (4). BMP-6 mRNA is expressed selectively in the roofplate adjacent to the forebrain and in cells adjacent to the

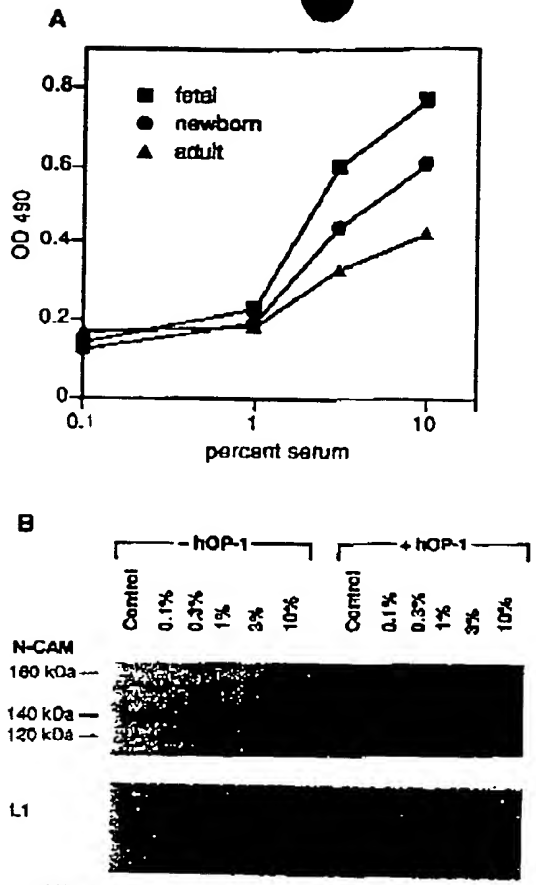


FIG. 4. Effect of serum on N-CAM and L1 expression in NG108-15 cells. *a*, NG108-15 cells were cultured in 96-well trays containing serum-free medium in the absence and presence of the indicated concentrations of fetal bovine serum, calf serum, and adult bovine serum. After 3 days cells were subjected to ELISA for N-CAM determination. Shown are the optical densities from a single experiment that was repeated three times with similar results. *b*, NG108-15 cells were cultured for 3 days in 6-well trays in serum-free medium supplemented with 0 (control) to 10% fetal bovine serum in the absence and presence of 40 ng/ml hOP-1. Cells were harvested and subjected to immunoblot analysis for N-CAM and L1. Shown is a representative autoradiograph from an experiment that was repeated three times with similar results.

floor plate along the anterior-posterior neuraxis (6, 29). BMP-4, BMP-5, and BMP-6 mRNA are not detected in the brains of 2-week post-natal and adult mice; however, OP-1 (BMP-7) mRNA is present in both (2), suggesting that OP-1 may also play a role in the adult nervous system.

Other members of the TGF-β superfamily are also active in mesenchymal and neural cells. Activin induces N-CAM and stimulates chondrogenesis in developing limb buds (13), but also induces somatostatin immunoreactivity in ciliary ganglion neurons (30) and is a neuronal survival factor (31). Dorsalin-1 is a novel member of the *dpp*/Vg-related subfamily that shows 55% sequence identity with BMP-4 and is selectively expressed in dorsal regions of the neural tube (3). Dorsalin-1 promotes the differentiation and migration of neural crest cells and inhibits the differentiation of motor neurons (3). Dorsalin-1 also induces alkaline phosphatase activity in W-20-17 osteoblast cells (3), demonstrating that like OP-1, BMP-4, and activin, dorsalin-1 has biological activity in bone and in brain.

It is unknown how most members of the TGF-β superfamily produce their morphogenetic effects. We hypothesize that some of these morphogens act in mesenchymal and neural tissue by inducing the expression of IgCAMs at critical periods in development and tissue repair. OP-1 stimulates endochondral bone formation (16), and N-CAM is transiently expressed in osteoblasts during intramembranous and endochondral bone formation (32). It is not yet known whether the induction of IgCAMs mediates the osteogenic actions of the BMPs or the neural actions of dorsalin and the BMPs. However, it is possible that a conserved signaling pathway couples some members of the TGF-β superfamily to the induction of IgCAMs in mesenchymal and neural cells, with different, tissue-specific effects.

TABLE 1

Effect of growth factors and cytokines on N-CAM expression

NG108-15 cells were cultured in 96-well trays containing serum-free medium in the absence and presence of 40 ng/ml of the indicated growth factors and cytokines. After 3 days cells were subjected to ELISA for N-CAM determination. Optical densities for control values determined on the same tray were normalized to 1, and values for the morphogen-treated cells were expressed relative to control values (relative OD). Shown are the mean ± S.E. relative OD values for the indicated number of experiments (parentheses). Except for the BMPs, similar results were obtained when the factors were tested at their half-maximal biological activity (ED_{50}) and one tenth of ED_{50} (as listed in the manufacturers specification sheets). All growth factors and cytokines used were recombinant human except TGF-β2 (porcine), TGF-β3 (chicken), and TGF-β5 (*Xenopus*).

Growth factor (40 ng/ml)	Relative OD, mean ± S.E. (*n*)
BMP-2	6.01 ± 0.66 (5)
BMP-4	6.38 ± 1.85 (5)
BMP-5	4.93 ± 2.03 (2)
BMP-6	4.43 ± 0.54 (5)
OP-1 (BMP-7)	6.30 ± 1.69 (4)
Activin-A	0.99 ± 0.08 (5)
Transforming growth factor-β1	1.05 ± 0.02 (3)
Transforming growth factor-β2	0.91 ± 0.07 (4)
Transforming growth factor-β3	1.06 ± 0.30 (3)
Transforming growth factor-β5	0.89 ± 0.07 (3)
Inhibin-A	1.01 ± 0.06 (5)
Nerve growth factor (2.5 S)	1.02 ± 0.06 (6)
Fibroblast growth factor-4	0.98 ± 0.38 (2)
Epidermal growth factor	0.95 ± 0.10 (4)
Platelet-derived growth factor-AA	1.09 ± 0.04 (2)
Platelet-derived growth factor-BB	1.09 ± 0.12 (2)
Platelet-derived growth factor-AB	1.08 ± 0.25 (2)
Interleukin-1α (IL-1α)	0.89 ± 0.09 (2)
Interleukin-1β	0.81 ± 0.00 (1)
Interleukin-2	1.02 ± 0.02 (2)
Interleukin-3	1.00 ± 0.23 (2)
Interleukin-4	1.09 ± 0.06 (2)
Interleukin-5	0.93 ± 0.13 (2)
Interleukin-6	0.80 ± 0.10 (2)
Interleukin-7	0.80 ± 0.02 (2)
Interleukin-8	0.83 ± 0.11 (2)
Interleukin-9	0.91 ± 0.10 (2)
Interleukin-10	0.91 ± 0.07 (2)
Interleukin-11	0.80 ± 0.03 (2)
Interleukin-1 ra	1.04 ± 0.09 (2)
Interleukin-γ	1.00 ± 0.07 (2)
GRO α/melanoma growth-stimulating activity	1.06 ± 0.16 (2)
Leukemia-inhibitory factor	1.19 ± 0.05 (2)
Oncostatin M	0.78 ± 0.20 (2)
Tumor necrosis factor-α	0.90 ± 0.12 (2)
Tumor necrosis factor-β	0.83 ± 0.06 (2)
Macrophage inflammatory protein-1α	0.89 ± 0.13 (2)
Macrophage inflammatory protein-1β	0.92 ± 0.17 (2)
Monocyte chemotactic protein-1	0.85 ± 0.30 (2)
RANTES (regulated upon activation, normal T-cell expressed and presumably secreted)	0.87 ± 0.07 (2)

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